Picosecond and Nanosecond Geminate Recombination of Myoglobin with CO, O_2, NO, and Isocyanides

Karen A. Jongeward,[†] Douglas Magde,*[†] Douglas J. Taube,[†] James C. Marsters,[†] Teddy G. Traylor,*[†] and Vijay S. Sharma[‡]

Contribution from the Departments of Chemistry and Medicine, University of California at San Diego, La Jolla, California 92093. *Received April 13, 1987*

Abstract: With use of an amplified colliding-pulse mode-locked ring dye laser, the recombination kinetics of small ligands binding to sperm whale and elephant myoglobins have been studied on the picosecond time scale. Nanosecond experiments were performed as well. On the picosecond time scale, all ligands investigated except CO show a substantial amount of rebinding. Picosecond rebinding of the isocyanides to iron is surprising as they are isoelectronic with CO and might be expected to show the same or similar recombination kinetics; the difference may be due to the greater Lewis basicity of the isocyanides. All ligands, again excepting CO and sometimes NO, also undergo recombination from geminate pairs that is a thousand times slower and occurs on the nanosecond time scale. We propose that the geminate pair exists in two very different configurations: the contact pair, or fast-reacting form, in which the ligand remains within a few angstroms of the iron atom and the separated pair, or slow-reacting form, in which the ligand wanders throughout the so called "pocket". Although there could be some evolution of the slow-reacting form over time, we have no evidence for multiple, distinct versions of the separated pair. Since oxygen and the much larger *tert*-butyl isocyanide react quite similarly on both time scales, we must suppose that the "steric effects" invoked to account for the low overall combination rate of isocyanide reaction with myoglobin occur very early when the ligand first approaches and enters the protein. Comparison of NO rebinding in whale and elephant myoglobins, which differ in the distal environment of the heme, allows us to begin to explore the properties of the contact pair. There appear to be two distinguishable forms of the contact pair in whale myoglobin, from which we infer that the contact pair can be a rather well defined chemical complex.

The mechanisms involved in binding neutral ligands to hemes and hemoproteins have been investigated intensively in recent years. Not only are such processes basic to enzyme biochemistry, but they also raise fundamental issues about the reactivity of transition metals. The combination reaction of a ligand to a coordinatively unsaturated metal is extremely facile. Fortunately, it can be studied conveniently by photodissociating the ligand and observing recombination. A number of pioneering nanosecond[1,2] and picosecond[3-5] studies have revealed concentration-independent return of ligands to hemoproteins that is quite efficient in general. The minimum reaction scheme necessary to accommodate such recombination from geminate pairs is

$$\mathrm{FeL} \rightleftharpoons [\mathrm{Fe}\text{--}\mathrm{L}] \rightleftharpoons \mathrm{Fe} + \mathrm{L} \qquad (1)$$

Kinetic studies in frozen glasses at low temperatures[6-8] reveal a whole series of intermediates. This, in combination with the observation of both picosecond and nanosecond processes under ambient conditions, suggests that the geminate pair [Fe--L] may exist in multiple forms.

In order to establish the kinetics of ligation over all chemically meaningful time scales, we have undertaken picosecond, nanosecond, and microsecond flash photolysis studies of the reaction of NO, O_2, CO, and selected isocyanides with sperm whale myoglobin (WhMb) and elephant myoglobin (ElMb). To interpret our measurements, we must invoke a minimum of two very distinct forms of the geminate pair [Mb--L]. One we will write $[\mathrm{Mb}\text{–}\mathrm{L}]_c$ and term the "contact pair". It reacts with a rate constant near 5×10^{10} s^{-1}. (Under some conditions, our data suggest that there are two distinguishable variations of the contact pair, which suggests that the contact pair may exist as one or more distinct complexes.) The other we will write $[\mathrm{Mb}\text{---}\mathrm{L}]_s$ and term the "separated pair". It reacts with a rate constant near 10^7 or 10^8 s^{-1}. We attribute this process to a ligand that wanders more or less randomly throughout the heme pocket before reforming the contact pair that is the immediate precursor to bond formation.

Experimental Procedures

Materials. Lyophilized sperm whale myoglobin was purchased from Sigma and used without further purification except for degassing with argon to remove oxygen. Sodium dithionite, purchased from Virginia Chemical Co., and bis-tris [bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane], purchased from Behring Diagnostics, were used as received. Argon (Linde) was passed through an oxygen scrubber (American Scientific Product) and nitric oxide (Matheson) was passed through a 10 by 200 mm column of Ascarite (Aldrich) prior to use. The *tert*-butyl isocyanide, purchased from Aldrich, was used as received. Methyl and ethyl isocyanide were prepared from the corresponding formamides by literature procedures.[9] All of the isocyanides were stored at 0 °C.

Aqueous buffer was prepared to give a 0.1 M bis-tris and 0.1 M NaCl solution. The acidity was adjusted by the addition of small amounts of hydrochloric acid to give the desired pH. Unless specifically stated, the pH of all samples is 7.0.

Methods. The isocyanide myoglobin samples were made by degassing approximately 10 mL of pH 7.0 bis-tris buffer with argon. After the addition of 25 μL of the isocyanide, the solution was shaken for 5 min. Next, 5 mL of the isocyanide buffer solution was cannulated to a degassed tube containing 5 mg of whale myoglobin. This solution was carefully mixed and cannulated to a degassed quartz cuvette. The myoglobin was then reduced with 1 μL of degassed, saturated aqueous sodium dithionite solution in 0.05 M sodium borate.

For the preparation of the oxymyoglobin samples, a Sephadex G-25 column was kept under a nitrogen atmosphere and equilibrated overnight with deoxygenated 0.1 M pH 7.0 bis-tris buffer. Concentrated solutions of ferric myoglobin were deoxygenated by passing nitrogen over them for 30 min and reduced by the addition of a few drops of a concentrated sodium dithionite solution. The sample of myoglobin was applied to the column and eluted with deoxygenated buffer. All operations were carried

[†] Department of Chemistry.
[‡] Department of Medicine.

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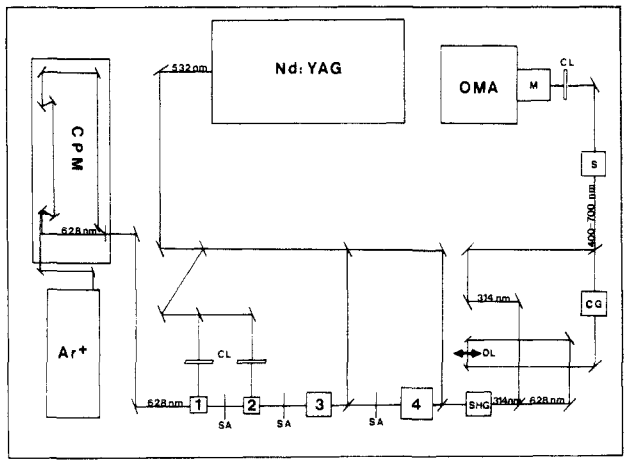

Figure 1. Schematic of the CPM ring laser and dye amplifier used in the picosecond experiments: CL, cylindrical lens; 1–4, gain stages; SA, saturable absorber jets; SHG, second harmonic generator; DL, delay line; CG, continuum generator; S, sample cell; M, monochromator.

out anaerobically. The eluted myoglobin samples were then diluted with the buffer to give an absorbance of about 1.0 in the sample cell. The final solutions were equilibrated with pure oxygen.

For the NO experiments, 4 mg of sperm whale myoglobin was placed in the bottom of a tonometer. With the tonometer held horizontal, 4 mL of buffer solution was added to the bulb. Care was taken so as not to wet the myoglobin. The buffer was degassed with several freeze–pump–thaw cycles and placed under argon. The myoglobin was then gently dissolved and reduced with 1 μL of the aqueous sodium dithionite solution. The myoglobin solution was slowly stirred under a stream of nitric oxide for an hour. Excess NO was needed to remove the remaining sodium dithionite. The solution was then cannulated into a degassed 2-mm quartz cuvette.

In all of the samples, the concentration of myoglobin was adjusted to give Soret absorptions of close to 1.0 in a 2-mm quartz cuvette, approximately 3×10^{-5} M.

Static absorption spectra were taken on Perkin-Elmer 124 and Kontron Uvicon 810 spectrophotometers before and after the experiments to check for degradation during the runs.

Instrumental Procedures. In the picosecond experiments, a Spectra-Physics Model 2020 CW argon ion laser operating "all-lines" is used to pump a colliding-pulse mode-locked ring dye laser.[10] The gain medium is rhodamine 590 chloride in ethylene glycol and the saturable absorber is a concentrated solution of DODC Iodide, also in ethylene glycol. Both dyes were purchased from Exciton. The time duration of the output pulses is measured with an autocorrelator and is found to be 0.07 ps. The pulses, centered at 628 nm, are essentially transform limited. The pulse energy is about 0.1 nJ per pulse and the repetition rate is 72 MHz. One-half of the pulses are sent to an autocorrelator and an electronic synchronization circuit for the amplifier. The remaining pulses are sent to a four-stage dye amplifier. The experimental arrangement is shown in Figure 1.

The amplifier consists of four dye cells separated by three saturable absorber jets. The first three cells contain decreasingly concentrated solutions of rhodamine 640 perchlorate in a 2% aqueous Ammonyx solution. The fourth cell contains a dilute solution of sulforhodamine, also in Ammonyx solution. The rhodamine dyes and the Ammonyx were purchased from Exciton. The saturable jets contain solutions of malachite green in ethylene glycol; that dye was purchased from Eastman-Kodak.

The amplifier is pumped by the 532-nm second harmonic of a Quanta-Ray DCR-2A Nd:YAG laser. The YAG pulse duration is 4 ns, the repetition rate is 10 Hz, and the energy used is about 250 mJ per pulse. Overall gain for the amplification of the femtosecond beam is 10^7. The output energy is 1.1 mJ per pulse with approximately 10% amplified spontaneous emission. Typical energy fluctuation is about 5% peak-to-peak.

The picosecond experiments are pump-probe measurements of the transient absorption measured for various settings of an optical delay line. In order to generate a pump, or photolysis, beam, the amplified fundamental beam is reduced in size to about 1 mm in diameter and passed through a 1 mm thick $LiNbO_3$ doubling crystal, producing 65-μJ pulses at 314 nm. The amount of UV light generated is kept constant to within ±2 μJ by changing the position of the doubling crystal. The remaining red light is sent to a delay line that has up to a 10 ns delay range with subpicosecond resolution. After the delay, the beam is tightly focused into a 1-cm water cell to create white light by self-phase modulation. Colored glass filters are used to select the appropriate wavelength as the probe beam. The region from 400 to 480 nm is chosen for these recombination studies. Next, the probe light is divided into two equally intense beams. One beam is used as the reference in a double beam arrangement, passing through a region of the sample that is not illuminated by the pump beam. The other probe beam is recombined with the photolysis beam and they are colinearly focused to a 0.5-mm spot at the sample cell. The time durations of the pump and probe pulses at the sample cell are less than 1 ps. The cell is held in a motorized mount that moves the cell enough to ensure that each laser shot sees fresh solution. The sample cells are 2 mm thick Precision quartz cuvettes fitted with air-tight rubber septa. After the sample, the probe beam and the reference beam are focused with a cylindrical lens onto different portions of the entrance slit of a spectrograph and then imaged onto the vidicon detector of a Princeton Applied Research optical multichannel analyzer OMA-II. The spectral region recorded totals 80 nm. The data are transferred from the OMA to a Celerity 1260D computer for processing.

Rate constants over the range 3×10^{11} to 5×10^8 s^{-1} are readily measured with the picosecond apparatus operating in the configuration described above. Rate constants over the range 10^8 to 10^5 s^{-1}, or even slower, are measured with a nanosecond apparatus described next.

For the nanosecond experiments, a Lumonics 861T XeCl excimer laser is used to pump a Lumonics dye laser. The gain medium is coumarin 540 in methanol. The output pulses at 540 nm have a duration of 4 ns at a 1-Hz repetition rate and are used as the photolysis source. A pulsed Xe flashlamp is used to probe the sample absorbance. A small Bausch and Lomb monochromator restricts the probe light to a 5 nm bandwidth before the sample. After the sample, the probe light is passed through a blue Corning glass filter, focused onto the slit of a J-Y H20 monochromator, and detected by an IP28 photomultiplier. The photocurrent is amplified with a Pacific Precision AD-6 amplifier and the resulting signal is sent to a Biomation 6500 interfaced to a Z-80 microprocessor. Two hundred shots are averaged per run. Data are transferred to a Celerity 1260D computer for analysis.

Results

We find both picosecond and nanosecond reactions of the geminate pairs (as well as millisecond bimolecular recombination) in the same systems. Therefore, we require an analysis in terms of a four-state model. The bound species, MbL, is represented as A; the short-lived (contact) geminate pair, $[Mb–L]_c$, as B; the longer lived (separated) geminate pair, $[Mb\cdots L]_s$, as C; and the two entirely separated free species, Mb + L, are represented as D.

$$A \underset{k_{BA}}{\overset{h\nu}{\rightleftharpoons}} B \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} C \underset{k_{DC}}{\overset{k_{CD}}{\rightleftharpoons}} D \qquad (2)$$

The initial efficiency (quantum yield) for the photolytic formation of B will be termed Γ_B. Since this value is not known exactly for any case, but is now known to be close to unity, we shall assume $\Gamma_B = 1.0$ in all calculations. We will comment on the adequacy of this approximation in the Discussion.

The rate constant for the disappearance of the contact geminate pair, B, is $k_{g,ps} = k_{BA} + k_{BC}$. The return rate to reform the contact pair from the separated pair, k_{CB}, can be ignored because it is approximately 1000 times slower than k_{BA} or k_{BC}. Thermal dissociation of A is also totally negligible on the picosecond time scale. The fraction of species B that go on to form the long-lived separated pair C is Γ_C, given by the ratio $\Delta A(t = \infty)/\Delta A(t = 0)$, where $(t = \infty)$ refers to a time as which the fast recombination has "leveled off"—a few hundred picoseconds—and $(t = 0)$ implies an extrapolation to an initial value. The rate constants k_{BA} and k_{BC} can be determined.

$$\Gamma_C = k_{BC}/(k_{BA} + k_{BC}) \qquad (3)$$

$$k_{BA} = k_{g,ps}(1 - \Gamma_C) \qquad (4)$$

$$k_{BC} = k_{g,ps}\Gamma_C \qquad (5)$$

The rate constant for the disappearance of the separated geminate pair, C, is given by $k_{g,ns} = k_{CD} + k_{CB}(1 - \Gamma_C)$. The backwards rate, k_{DC}, is bimolecular and may be ignored if con-

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Table I. Rate Constants and Lifetimes for the Picosecond Geminate Recombination of O_2, Isocyanides, and NO to Myoglobin

sample	$k_{g,ps}$ (s^{-1})	$\tau_{g,ps}$ (ps)	Γ_C	k_{BA} (s^{-1})	k_{BC} (s^{-1})
$WhMbO_2$	$2.9 \pm 0.4 \times 10^{10}$	40 ± 5	0.52 ± 0.10	$1.4 \pm 0.4 \times 10^{10}$	$1.5 \pm 0.6 \times 10^{10}$
WhMbCNMe	$5.3 \pm 0.9 \times 10^{10}$	20 ± 3	0.76 ± 0.06	$1.3 \pm 0.4 \times 10^{10}$	$4.0 \pm 0.8 \times 10^{10}$
WhMbCNEt	$2.1 \pm 0.3 \times 10^{10}$	48 ± 7	0.74 ± 0.05	$5.5 \pm 1.3 \times 10^{9}$	$1.6 \pm 0.2 \times 10^{10}$
WhMbCNtBu	$3.4 \pm 0.2 \times 10^{10}$	30 ± 2	0.25 ± 0.02	$2.6 \pm 0.2 \times 10^{10}$	$8.0 \pm 2.8 \times 10^{9}$
WhMbNO					
pH 7.0	$1.6 \pm 0.9 \times 10^{11}$	6 ± 3	≈ 0	$1.6 \pm 0.9 \times 10^{11}$ [a]	≈ 0
	$1.4 \pm 0.7 \times 10^{10}$	71 ± 36	0.15 ± 0.02	$1.2 \pm 0.6 \times 10^{10}$	$2.0 \pm 1.0 \times 10^{9}$
pH 6.1	$2.7 \pm 0.1 \times 10^{10}$	37 ± 2	0.15 ± 0.01	$2.3 \pm 0.1 \times 10^{10}$	$4.0 \pm 1.4 \times 10^{9}$
pH 5.8	$1.7 \pm 0.1 \times 10^{10}$	59 ± 4	0.17 ± 0.02	$1.4 \pm 0.1 \times 10^{10}$	$3.0 \pm 1.4 \times 10^{9}$
ElMbCNEt	$5.5 \pm 0.7 \times 10^{10}$	18 ± 2	0.49 ± 0.05	$2.8 \pm 0.4 \times 10^{10}$	$2.7 \pm 0.4 \times 10^{10}$
ElMbNO	$11.1 \pm 0.1 \times 10^{10}$	9 ± 1	≈ 0	$1.1 \pm 0.1 \times 10^{11}$ [a]	≈ 0

[a] $k_{B'A}$.

Table II. Rate Constants and Lifetimes for the Nanosecond Geminate Recombinations of O_2 and Isocyanides to Myoglobin

sample	$k_{g,ns}$ (s^{-1})	$\tau_{g,ns}$ (ns)	Γ_D	k_{CB} (s^{-1})	k_{CD} (s^{-1})
$WhMbO_2$	$1.10 \pm 0.17 \times 10^{7}$	90 ± 14	0.80 ± 0.01	$0.46 \pm 0.12 \times 10^{7}$	$0.88 \pm 0.16 \times 10^{7}$
WhMbCNMe	$2.45 \pm 0.11 \times 10^{7}$	40 ± 5	0.24 ± 0.08	$7.75 \pm 2.12 \times 10^{7}$	$0.59 \pm 0.20 \times 10^{7}$
WhMbCNEt	$7.6 \pm 1.0 \times 10^{7}$	13 ± 2	0.16 ± 0.08	$2.45 \pm 0.62 \times 10^{8}$	$1.22 \pm 0.64 \times 10^{7}$
WhMbCNtBu	$0.75 \pm 0.20 \times 10^{7}$	135 ± 36	0.48 ± 0.10	$0.52 \pm 0.17 \times 10^{7}$	$0.36 \pm 0.12 \times 10^{7}$
$ElMbO_2$	$0.5 \pm 0.04 \times 10^{7}$	200 ± 16	0.82 ± 0.01	$0.09 \pm 0.01 \times 10^{7}$ [a]	$0.41 \pm 0.03 \times 10^{7}$

[a] This number was not calculated by using the full eq 7 for k_{CB} as Γ_C could not be determined for this complex. Here, $k_{CB} = k_{g,ps}(1 - \Gamma_D)$, and so is a lower limit to the actual rate constant.

centrations are adjusted so that the pseudo-first-order rate of the nongeminate rebinding is at least 10 times slower than k_{CD} and k_{CB}. The fraction of molecules that go on from C to form D, denoted Γ_D, is obtained directly from the nanosecond data as $\Delta A(t = \infty)/\Delta A(t = 0)$, where $(t = \infty)$ refers to a time of several hundred nanoseconds at which recombination from all geminate pairs is complete but bimolecular recombination has not commenced, and $(t = 0)$ implies an extrapolation of the nanosecond decay to an initial value, ignoring any initial transient associated with the fast, picosecond process. If, as is the case here, k_{CB} and k_{CD} are much slower than k_{BA} and k_{BC}, the rate constants are calculated as

$$\Gamma_D = k_{CD}/[k_{CD} + k_{CB}(1 - \Gamma_C)] \quad (6)$$

$$k_{CB} = k_{g,ns}(1 - \Gamma_D)/(1 - \Gamma_C) \quad (7)$$

$$k_{CD} = k_{g,ns}\Gamma_D \quad (8)$$

The analysis may be tested against known steady state (or millisecond pulsed) dissociation yields, termed Q, since the model predicts $Q = \Gamma_B\Gamma_C\Gamma_D$.

The transient spectra in the picosecond experiments, Figures 2–4, are calculated as follows

$$\Delta A_\lambda = \log [(M_{unp}/M_{unp\text{-}ref})/(M_p/M_{p\text{-}ref})] \quad (9)$$

where M_{unp} is the transmittance of the unpumped sample, M_p is the transmittance of the pumped sample, and $M_{unp\text{-}ref}$ and $M_{p\text{-}ref}$ are the transmittances of the reference beam. A background curve is subtracted from each signal and reference track before the change in absorption is calculated.

Each system was studied on three to six separate occasions, over a period of several months. The results were reproducible and the figures presented here are typical examples of the results obtained. In each experimental run, data were collected at 15 to 20 different time delays and all points were used in the calculation of the rate constants. For clarity, in Figures 2–4, only a few of the experimental curves are shown.

Figure 2 shows the transient difference spectra for the picosecond recombination of O_2 to sperm whale myoglobin at delay times of 2, 10, 75, 100, and 150 ps. The negative absorbance, or bleaching, at about 420 nm is due to the disappearance of the ground-state six-coordinate species as the O_2 is photolyzed from the iron. The positive absorbance at about 440 nm is attributed to absorption by the five-coordinate deligated species. The inflection at about 440 nm is not real but is an experimental artifact caused by the detection system. The experimental curves have been smoothed once to reduce noise fluctuations. The amount of photolysis light was reduced to 30 μJ for this sample as the

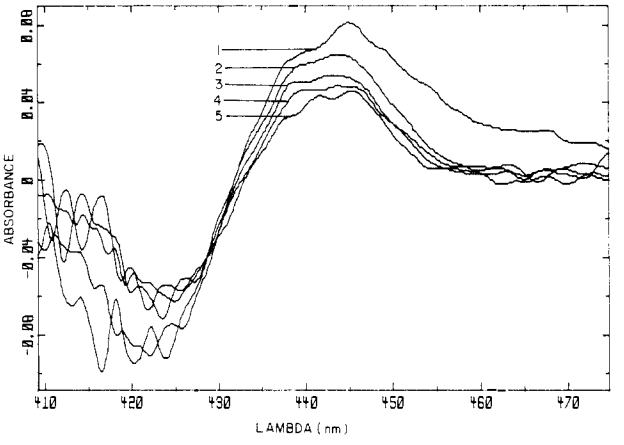

Figure 2. Picosecond transient difference spectra of $WhMbO_2$. The change in absorbance is plotted versus wavelength as a function of time delay between the pump and probe pulses. The numbers 1–5 label the curves obtained at 2, 10, 75, 100, and 150 ps after photolysis. Photolysis energy was 30 μJ at 314 nm. The inflection at about 440 nm is an experimental artifact.

MbO_2 photodegrades rather easily under UV excitation. In the kinetic plot, the decay of the induced absorbance changes is best fit with a single exponential function decaying as $k_{g,ps}$ plus a constant offset associated with Γ_C. The rates and geminate lifetime are listed in Table I.

We have also studied the nanosecond recombination from geminate pairs of O_2 with whale myoglobin. The rates and geminate lifetime are listed in Table II.

Picosecond experiments on the recombination of O_2 to elephant myoglobin were attempted, but due to the even greater photodegradation of this sample after UV excitation and the limited quantities available, quantitative results were not obtained. Although it was not possible to determine a rate constant or lifetime for the elephant sample, it was clear that the initial intensity of the transient absorption signal decreased approximately 40% in the first 150 ps after photolysis. Unlike the picosecond measurements which use UV excitation and record absorption changes at only one time delay for each laser shot, the nanosecond procedure employs excitation in the green and samples all time delays on each laser photolysis pulse. Consequently, it was possible to characterize the nanosecond geminate process quantitatively in $ElMbO_2$, and those results are listed in Table II.

The picosecond recombination of methyl, ethyl, and *tert*-butyl isocyanides to myoglobin was also studied. Figure 3 shows the

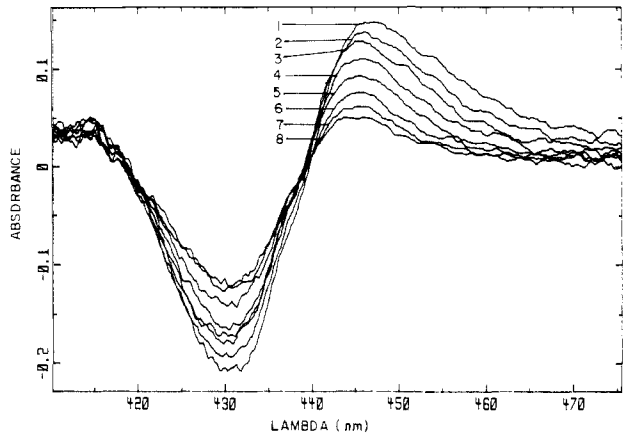

Figure 3. Picosecond transient difference spectra of WhMbCNtBu. The change in absorbance is plotted versus wavelength as a function of time delay between the pump and probe pulses. The numbers 1–8 label the curves obtained at 2, 4, 6, 8, 12, 13, 75, and 175 ps after photolysis. Photolysis energy was 65 μJ at 314 nm.

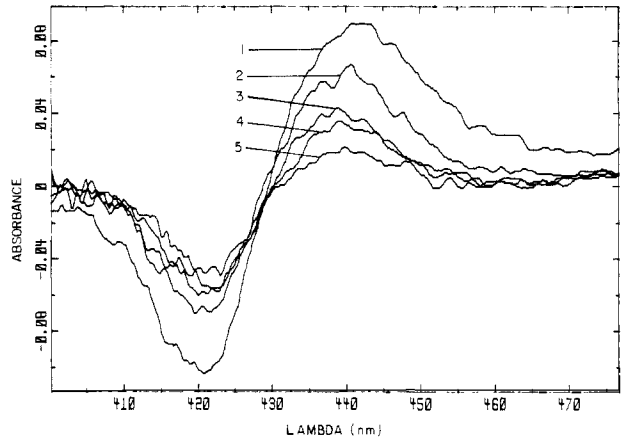

Figure 4. Picosecond transient difference spectra of WhMbNO. The change in absorbance is plotted versus wavelength as a function of time delay between the pump and probe pulses. The numbers 1–5 label the curves obtained at 0, 10, 30, 50, and 100 ps after photolysis. Photolysis energy was 60 μJ at 314 nm.

transient difference spectra of *tert*-butyl isocyanide at delay times of 2, 4, 6, 8, 12, 13, 75, and 175 ps after photolysis. As in $WhMbO_2$, the experimental curves have been smoothed once. Notice the excellent isosbestic point obtained; this is typical of all the isocyanide measurements. Figure 5 shows a kinetic plot of the decay of the transient absorbance of *tert*-butyl isocyanide at λ_{max} as a function of delay time and demonstrates the excellent first-order kinetics that we observed. As in the O_2 case, for all three isocyanides there is a substantial disappearance of the transient absorption, which is attributed to rebinding of the ligand to the heme iron, on the picosecond as well as the nanosecond time scale. All picosecond decays for the isocyanides are well fit with single exponential functions. The picosecond rates and geminate lifetimes are listed in Table I. Figure 6 shows the nanosecond recombination spectrum of WhMbCNMe. All of the nanosecond isocyanide data are listed in Table II.

We have also studied the picosecond rebinding of ethyl isocyanide to elephant myoglobin. The results, shown in Table I, reveal a reduced Γ_C, a faster recombination, and increased values of both k_{BA} and k_{BC} than observed for WhMbCNEt.

There is only a little to be said about MbCO. Even though this laboratory has easily observed recombination from geminate pairs in HbCO,[11,12] due to instrumental limitations, we are unable

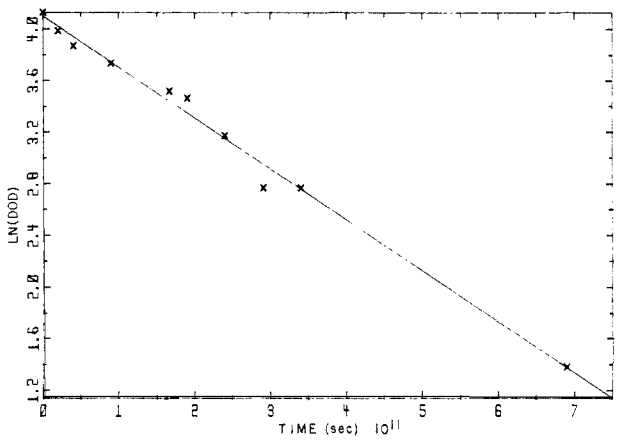

Figure 5. Kinetic plot for the rebinding of CNtBu to deoxy WhMb. The decay of the transient absorbance at λ_{max} is plotted as a function of delay time between the photolysis and the probe pulses.

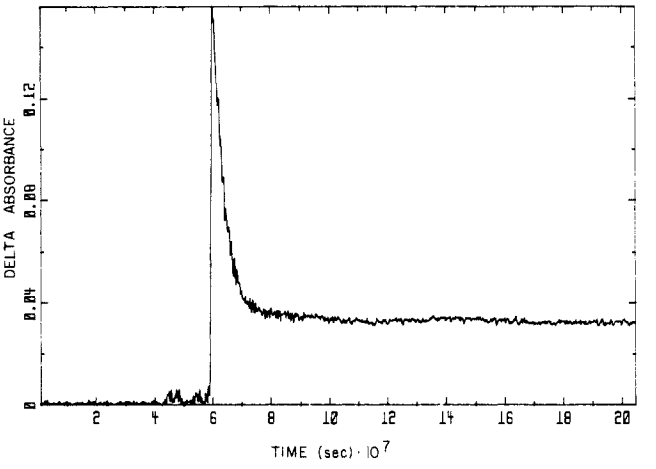

Figure 6. Nanosecond transient absorption kinetics of WhMbCNMe. The absorbance change at 440 nm is plotted versus delay time. Photolysis energy was 3 mJ at 540 nm.

so far to observe any geminate rebinding on any time scale in WhMbCO, despite several attempts on both instruments.

The rebinding of NO to WhMb reveals additional complexities. The transient spectra of WhMbNO is shown in Figure 4 at delay times of 0, 10, 30, 50, and 100 ps, and as in the previous figures, the experimental curves have been smoothed once. The picosecond transient bleaching is best fit with a biexponential curve. The slower of the picosecond processes occurs with a characteristic time comparable to that observed for the other ligands; in addition there is a rebinding process that is markedly faster. With use of the biexponential fit, it is found that the zero-time amplitude of the slow component is about twice that of the fast. (This amplitude ratio was reported backwards in the text of our previous communication,[13] although the fitted curves displayed with the data were calculated and drawn correctly.) At 500 ps, the signal has decreased to a constant value with about 15% of the initial transient absorbance still remaining, which we associate with Γ_C. Unfortunately, we were unable to measure the extent of nanosecond recombination or determine a rate constant for the nanosecond process.

In order to understand the biphasic picosecond rebinding in WhMbNO, we investigated the effect of changing pH and also compared the WhMbNO with ElMbNO. Data for the whale–elephant comparison were reported previously.[13] A single exponential fit is adequate to describe ElMbNO: the bleaching decays very rapidly as the NO rebinds to the iron, and at 100 ps, the signal is indistinguishable from the noise.

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In order to interpret the NO recombination, we must add a new feature to our model. The simplest possibility consistent with the data is the following:

$$A \underset{k_{BA}}{\overset{h\nu}{\rightleftharpoons}} B \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} C \underset{k_{DC}}{\overset{k_{CD}}{\rightleftharpoons}} D \quad (10)$$

$$A \underset{k_{B'A}}{\overset{h\nu}{\rightleftharpoons}} B' \quad (11)$$

In brief, we imagine that there are two forms of the fast-combining contact pair, B and B', one of which recombines so efficiently, at least for NO, that it is effectively uncoupled from all other processes.

In the photolysis of ElMbNO, the only species formed is B'. In this case, Γ_C is 0 and $k_{B'A}$ is very large and equal to $k_{g,ps}$.

In treating the recombination of NO to whale myoglobin at pH 7.0, we assume a superposition of the process associated with B' in ElMbNO and the normal process observed with other ligands. Within experimental error, the fastest rate for WhMbNO is the same as that for ElMbNO. Therefore, in fitting the faster picosecond rebinding process, Γ_C is set to zero and $k_{g,ps} = k_{B'A}$. In the slower picosecond process, B may go on to form C or back to form A and $k_{g,ps}$ again equals the sum of the two rates, k_{BA} and k_{BC}. At 500 ps, Γ_C is 0.15. As 500 ps amounts to 80 lifetimes of B', the amount of B' left is negligible. The rates and lifetimes for the picosecond recombination of NO to both elephant and whale myoglobin are listed in Table I.

The recombination of NO to whale myoglobin was also studied as a function of pH. For pH below 7, the recombination becomes monophasic and the rates are nearly the same as the slow picosecond rate seen in the pH 7.0 study. At pH 6.1, the rates are again similar. In the pH 5.8 experiment, at 500 ps, about 85% of the photolyzed NO has rebound, leaving 15% still unrecombined as in the pH 7.0 study. This same value $\Gamma_C = 0.15$ was observed at every pH. At pHs more basic than 7.0, the rate of recombination remains biphasic.

In all of the above picosecond experiments, there was a spectral evolution within the first 5 to 10 ps. At time $t = 0$, there was a broad red absorption in the transient difference spectra, and in all cases the absorbance change was approximately 0.025 at 470 nm. This red absorption has been seen in both myoglobin and hemoglobin after photolysis of O_2 and CO and has been attributed to an "excited state".[14-17]

Discussion

It has been known for more than 15 years that the quantum yield for light-induced dissociation of ligands from heme proteins to form free pairs in solution varies widely with ligand and protein.[18] In MbCO, it is near 95% and in MbNO it is approximately 0.1%. Our measurements confirm that this variation is not due, except perhaps to a very small extent, to differences in the *initial* photolysis yield. Rather, a five-coordinate, out-of-plane iron is formed "instantaneously" with a yield that is close to unity. By "instantaneously", we mean certainly no more than a few picoseconds, that is, much faster than any of the kinetic processes we are concerned with in this report. The widely varying dissociation yields observed for different ligands on millisecond time scales are due primarily to the fact that significant amounts of recombination from geminate pairs take place on the picosecond and nanosecond time scales before the partners have time to diffuse apart.

Since the time scale for the formation of the deoxy heme species, with the iron atom out-of-plane, was formerly considered so uncertain that it could be anything from subpicoseconds to microseconds, it is essential to stress that we now consider the issue to have been settled by picosecond and subpicosecond studies, although others might still disagree. Transient Raman studies by Friedman et al.,[19] using 25-ps pulses at 435 nm, found that after photodissociation of both CO and O_2 from hemoglobin, the frequency of the iron–proximal histidine stretch in the spectrum of the deoxy transient was identical at 25 ps and 10 ns, while earlier work by the same group had established that the 10-ns form was essentially the deoxy form. This constitutes very strong evidence that the deoxy transient with the iron out-of-plane is fully formed within the time duration of their pulse, less than 25 ps. Using 250-fs pulses at 307 nm, Martin et al.[20] carried out a detailed examination of the formation of the geminate pair following photolysis of CO from protoheme and hemoproteins, including MbCO. They concluded that the deoxy species apppears in about 0.35 ps, with further relaxation to the equilibrated high-spin ground state of the geminate pair occurring in about three picoseconds. This relaxation to the high-spin ground state of the deoxy species is our interpretation of the spectral changes at 470 nm that we observed occurring in the first few picoseconds. All of the analysis below is concerned with processes that take place after this formation of the ground-state geminate pair. The processes we are watching at 420 and 440 nm take place over times ranging from tens to thousands of picoseconds. We are convinced that we are not observing significant amounts of excited-state relaxation at those wavelengths.

Even though we believe that the deoxy form is present within at most a few picoseconds, we must concede that it is not exactly the form that is observed for the equilibrium deoxy species at long times. An interesting phenomenon appears when transient difference spectra are overlapped with static difference spectra. (In the static spectrum, the change in absorbance is calculated as the spectrum of Mb, in aqueous solution but with no ligand added, minus the spectrum of MbL.) In all cases studied, the maxima of the bleachings overlap exactly, but the maxima of the absorptions from the five-coordinate species do not, the static peak being 3 to 4 nm blue-shifted. This difference does not change on the picosecond time scale and, consequently, does not affect the calculation of the rebinding kinetics. This blue-shift of the static peak has been seen by several other workers in similar systems.[14,16,20,21] Preliminary work with a monochelated protoheme also shows a 3-nm blue-shift of the static deoxy peak, suggesting that the shift is a function of the heme and not caused by protein conformational changes. Additional experiments are in progress to clarify this phenomenon.

The spectral evolution we observe in transient absorption spectra taken over a few hundred picoseconds following photolysis of MbNO, MbO_2, MbCNMe, MbCNEt, and MbCN-*t*-Bu shown in Figures 2–4 makes it quite clear that ligands return by very rapid processes from the dissociated state to bound states. Similarly, the nanosecond measurements clearly monitor a process that is also recombination from geminate pairs. Concentration-dependent, bimolecular processes are only observed at much longer times; nor do protein conformational changes cause spectral changes in this time range in myoglobin. Since both processes were characterized in the same laboratory, frequently by measurements on the same samples, there seems to be little question that two well-behaved first-order return processes occur quite generally for ligands bound to ferrous myoglobin. The two processes occur in completely different time ranges; their relaxation rates differ by a factor of 1000.

In the first process, all ligands studied, except CO, recombine with iron(II) porphyrin in hemoproteins with rate constants k_{BA} exceeding 10^{10} s^{-1}. We note that CO is also anomalous in that its overall second-order rate constant for binding in model com-

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pounds and hemoproteins (about 6×10^6 to 1×10^7 s^{-1} M^{-1}) is far below the diffusion limit, while the second-order rate constants for NO,[22] O_2,[23] isocyanides,[24] and imidazoles[25] for binding are all the same, approximately 10^8 s^{-1} M^{-1}, and apparently diffusion limited. It seems that only CO binding is activation controlled for the second-order combination reaction. Consequently, the three-state model may be used for some purposes in discussing CO binding;[26] the four-state model is needed in all other cases. Results for MbCO, of course, are still consistent with a four-state model characterized by small k_{BA} and, possibly, large k_{BC}.

The simplest physical model consistent with the two-step kinetic scheme involves distinguishing a contact pair, spaced a van der Waals distance apart, from a separated pair, which is still confined within the protein but much changed from the configuration needed to reform the iron–ligand bond. Photodissociation breaks the MbL bond, the iron moves out of plane, and any electronic or vibrational relaxation is completed within no more than a few picoseconds to form the contact pair B. At this point the ligand has moved no further than a few angstroms and rotated relatively little. This scheme is illustrated with an isocyanide ligand in eq 12. Return of the ligand to the iron is inhibited by the need to

$$\mathrm{MbCNR} \underset{k_{BA}}{\overset{h\nu}{\rightleftharpoons}} [\mathrm{Mb\text{–}CNR}]_c \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} [\mathrm{Mb—RCN}]_s \underset{k_{DC}}{\overset{k_{CD}}{\rightleftharpoons}} \mathrm{Mb} + \mathrm{RNC} \quad (12)$$

bring the iron into the plane of the porphyrin and accommodate possible spin changes, but even so k_{BA} remains surprisingly fast, except for CO. The ligand in state B may be stabilized by nearby amino acids, including the distal histidine. The ultrafast rebinding from the contact pair occurs in competition with rotation and translation of the ligand away from an orientation favorable to binding. This process is characterized by k_{BC}, which is also a very fast, picosecond process. During these tens of picoseconds, there can occur motion of nearby amino acid side chains, including some motion of the histidine, but the overall protein shape should not relax very much. Once the ligand has moved away from the configuration B to form the separated pair C, a relatively long (nanosecond) time is required to reform the contact pair through the process characterized by k_{CB}. Reformation of B is inhibited by the low probability of the ligand, now free to rotate and move throughout the pocket, executing a random walk to return to the vicinity of the iron and assume the correct orientation. This process is analogous to the intimate and solvent separated ion pairs in solvolysis reactions, documented with stereochemical and kinetic means by Winstein et al.[27]

$$\mathrm{RX} \rightleftharpoons [\mathrm{R^+X^-}] \rightleftharpoons [\mathrm{R^+||X^-}] \rightleftharpoons \mathrm{R^+} + \mathrm{X^-} \quad (13)$$

Some workers have proposed that in the separated pair C, the ligand moves not only around the pocket but throughout the entire volume of the protein. We do not favor that view and have previously given some arguments against that model.[12] However, in either case, the amount of ligand rebinding is determined by the ratio of k_{CB} to k_{CD}, the latter being the rate constant for escape to the solvent and formation of the solvent-separated pair.

The duration of the ligand's stay in the pocket, which determines k_{CD}, may be limited by the frequency of protein fluctuations which eventually open a channel allowing the ligand to exit. Such a process was analyzed in molecular dynamics calculations carried out be Case et al.[28] They found that a ligand with properties rather like O_2 wandered freely around the pocket until it escaped through a channel to the solvent. The rebinding process was forced to have zero probability. The one rate that could be calculated agreed very well with our result. They found $k_{CD} = 1.3 \times 10^7$ s^{-1}. Furthermore, nothing in their model predicts much variation among diatomic ligands of similar size, which is what we find. It is unclear what the simulation would predict for the bulky *tert*-butyl isocyanide ligand. Calculating k_{CD} for that ligand to compare with our data should be an interesting and relatively simple test of molecular dynamics models.

Our data along with published[29,30] overall kinetic and equilibrium constants allow a complete description of binding of dioxygen and *tert*-butyl isocyanide to sperm whale myoglobin, including the calculation of k_{AB}, the rate of thermal dissociation of the iron–ligand bond, which was neglected in eq 2 because of its small size compared to the photolysis rate. In the following equations, all rate constants are given in s^{-1} except for k_{DC}, which is given in s^{-1} M^{-1}.

$$\mathrm{MbO_2} \underset{1.4 \times 10^{10}}{\overset{28}{\rightleftharpoons}} [\mathrm{Mb\text{–}O_2}]_c \underset{4.6 \times 10^6}{\overset{1.5 \times 10^{10}}{\rightleftharpoons}} [\mathrm{Mb—O_2}]_s \underset{1.1 \times 10^8}{\overset{8.8 \times 10^6}{\rightleftharpoons}} \mathrm{Mb} + \mathrm{O_2} \quad (14)$$

$$\mathrm{MbCN\text{-}}t\text{-}\mathrm{Bu} \underset{2.6 \times 10^{10}}{\overset{11}{\rightleftharpoons}} [\mathrm{Mb\text{–}CN\text{-}}t\text{-}\mathrm{Bu}]_c \underset{5.2 \times 10^6}{\overset{8 \times 10^9}{\rightleftharpoons}} [\mathrm{Mb—CN\text{-}}t\text{-}\mathrm{Bu}]_s \underset{4 \times 10^3}{\overset{3.6 \times 10^6}{\rightleftharpoons}} \mathrm{Mb} + \mathrm{CN\text{-}}t\text{-}\mathrm{Bu} \quad (15)$$

The question arises whether the four-state model must be a serial reaction. Could the picosecond fast recombination and the nanosecond slow recombination occur in parallel? We believe that the serial model is more likely. We note that the measured k_{BA} are indeed very fast. It must be a contact pair that is involved. Whatever the details, this contact pair, B, seems to be so intimately related to the iron that we find it difficult to imagine how the slow reacting geminate pair, C, could react with the iron without passing through a state essentially similar to B.

Distal Effects and Ligand Basicity. The views hypothesized in the above model require a redefinition of notions of distal steric effects. Since k_{BA} is rather similar for *tert*-butyl isocyanide, methyl isocyanide, and O_2 (and somewhat larger for ethyl isocyanide and NO), there cannot be a large steric effect upon the actual bond making—certainly none that differentiates strongly based upon size or shape. Therefore, steric effects in myoglobin ligation must involve processes that occur at an earlier stage. However, the variations observed in k_{CB} do not correlate in any straightforward manner with ligand size either. Since it is the case that large isocyanides do have significantly lower overall bimolecular combination rates than does O_2, the external combination k_{DC} must be reduced. This may be seen in eq 14 and 15. In other words, differentiation based upon size occurs when the ligand enters the protein from the solvent. The traditional view[31] of steric effects acting upon bond making is very misleading and, in fact, incorrect. We will treat this issue in more detail elsewhere.

One of the surprises in this study is the very fast reaction of all the isocyanides, compared with the slow reaction of CO, even though both are singlet molecules. The fast reactions of O_2 and NO have often been attributed to spin-allowed processes in contrast to CO for which spin changes are required. If this were the entire story, one would not expect a fast reaction for the singlet isocyanides. One explanation is for a strong Lewis base such as an isocyanide or an imidazole to react with the iron in an acid–base reaction, producing a transient high-spin complex which subse-

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(27) Winstein's extensive work is most easily approached through a summary. See, for example: Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 3rd ed.; Haper and Row: New York, 1987; pp 346–349 and references therein.
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(31) See Traylor et al. (Traylor, T. G.; Tsuchiya, S.; Campbell, D.; Mitchell, M.; Stynes, D.; Koga, N. *J. Am. Chem. Soc.* **1985**, *107*, 604–614) for a discussion of various views of distal steric effects.

quently decays. Such processes might be expected for other

$$[Mb + :B] \rightleftharpoons [MbB]^* \xrightarrow{fast} MbB \qquad (16)$$

metal–ligand systems as well. It is evident that there are fundamental considerations in the reaction of Lewis base ligands with high spin metal systems which are not understood. Their study, as a function of metal spin state and ligand basicity, is in order.

Elephant Myoglobin and pH Effects. This myoglobin has a glutamine substituted for the distal histidine (E7), which has been implicated in proposed steric, polar, and hydrogen bonding effects on ligation in hemoproteins. We find a significant difference between WhMb and ElMb in k_{BA} for recombination from the contact pair. A single process is observed in ElMbNO, while biphasic recombination from the contact pair is observed for NO in WhMbNO. The faster process shows a k_{BA} almost identical with that in ElMbNO, while the slower process is ten times slower and is closer to the rate constant observed for other ligands. For simplicity, our data analysis treats the two processes as completely isolated from each other. However, it is more likely that there is rapid interconversion between B′ and B. In the case of NO, the rate of rebinding, k_{BA}, is fast enough to distinguish between them. In the case of ethyl isocyanide rebinding, the process appears monophasic at present experimental resolution but k_{BA} is almost three times as large in the elephant protein as in the whale. Γ_C in ElMbCNEt was substantially smaller than that in WhMbCNEt, meaning that the amount of rebinding from the contact pair has increased. This result is evidence that the distal amino acid does play a part in the fast rebinding from the contact pair, although since a fraction of ligands do go on to form the separated pair, the distal amino acid is not the only factor.

Our experiments show that as the pH is lowered below physiological pH, NO recombination in whale myoglobin is altered and the recombination process is no longer biphasic. Making the pH of the sample slightly acidic may change the tertiary conformation of the protein chain to a conformation of lower affinity so the photolyzed NO will take longer to recombine with the heme. This concept would be consistent with measurements of Friedman et al.,[32] who have recently studied various hemoglobins over a range of pH using picosecond time-resolved Raman spectroscopy. They found that the amount of CO rebinding was less at pH 6.2 and 3.0 mM IHP than at pH 8.2. In the presence of IHP, hemoglobin assumes a more T-like configuration, the transition state is destabilized, and the rate of ligand rebinding would be expected to decrease. Although myoglobin cannot exhibit the quaternary changes that contribute to the T-state, it may share whatever tertiary contributions may exist. We would expect such conformational changes to reduce both k_{BA} and $k_{B'A}$ but not necessarily equally. Probably, the faster would be slowed more. At the same time, there is no reason to expect any interconversion of B with B′ or any process forming C to change. The result would be to merge the recombinations from B and B′ until they are no longer resolvable. An experiment studying the effect of pH and IHP concentration on the picosecond rebinding of NO to hemoglobin might further define the pH effect and help to shed light on the similarities and differences between the fast reactions of the geminate pair in tetrameric hemoglobin and monomeric myoglobin.

Relation to Previous Work. In the following discussion, we assume that the recombination mechanism as well as the quantum yield for photodissociation of ligands from ferrous hemoproteins is independent of wavelength. This assumption has been made by many others and has positive support.[5,15,33-36] Although one might fear that subtle wavelength-dependent effects could occur in novel measurements on ultrafast time scales, we know of no plausible mechanisms. Changing the photolysis wavelength in our studies would be very difficult at the present time.

Our picosecond data for the recombination of O_2 to whale myoglobin are different from the results reported by Cornelius et al.[14] They report that, using 10 μJ, 8-ps, 353-nm pulses, they found no evidence of subnanosecond recombination in whale MbO_2. As can be seen from Figure 2, we find that there is a substantial amount of geminate rebinding. Our results are intermediate between those of Martin et al.,[15] who report a 100-ps time constant for 80% recombination of O_2 to whale myoglobin after photolysis with 20-μJ, 100-fs, 309-nm pulses, and those of Hutchinson et al.,[17] who saw about 50% recovery of photolyzed O_2 within 20 ps using 531-nm excitation. They are also broadly similar to results in the studies of O_2 recombination to a different protein, hemoglobin.[5,36]

Our observation of no picosecond recombination in MbCO contradicts the picosecond study of MbCO by Hutchinson et al.,[17] who report 19% rebinding in 20 ps and an additional 7% within 3 ns, but agrees with that of Martin et al.[20] It does agree with the report of Cornelius et al.;[14] but that work did not find any picosecond recombination for Mb with either CO or O_2.

Biphasic picosecond recombination in WhMbNO has not been reported previously,[42] but Cornelius et al.[21] did observe similar kinetics for NO geminate rebinding to human hemoglobin.

We are aware of no previous, direct picosecond measurements involving any isocyanides.

Recently, Gibson et al.[37] reported measurements of nanosecond rebinding to WhMb for all of the ligands we treat, including the isocyanides, which have been investigated only rarely.[38] In addition, they offered a comprehensive and critical summary of earlier nanosecond work. Our nanosecond results on the isocyanides, specifically $k_{g,ns}$, are in fairly good agreement with the k_{g1} values reported by Gibson. A somewhat greater discrepancy, a factor of 2.2, occurs for $WhMbO_2$.

Gibson et al.[37] emphasized correctly that it is important that kinetic models not only fit decay curves but also be consistent with measured quantum yields for the photolytic production of free pairs, D in our model. In all three of the isocyanides, it is quite clear that the two processes we have measured account for almost all of the recombination undergone by geminate pairs. There is little "missing amplitude". For methyl isocyanide, the quantum yield for dissociation into separated species D is given by Gibson et al.[37] as 0.17 ± 0.02, which is to be compared with our product $\Gamma_C\Gamma_D = 0.18 \pm 0.06$. We consider the agreement to be extremely good. In the ethyl isocyanide case, Gibson's Q is 0.065 ± 0.006 and our $\Gamma_C\Gamma_D = 0.12 \pm 0.06$. For *tert*-butyl isocyanide, Gibson's Q is 0.09 ± 0.01 and our $\Gamma_C\Gamma_D = 0.12 \pm 0.03$. In the ethyl and *tert*-butyl isocyanide cases, $\Gamma_C\Gamma_D$ may exceed Q slightly because we assume that Γ_B, the quantum efficiency for the photolytic formation of B, is unity, which may not be entirely accurate. However, in all of the isocyanides, there is agreement within experimental error.

There is a larger discrepancy in the case of MbO_2. The quantum yield Q for dissociation reported some time ago by Ainsworth et al.[39] was increased to $Q = 0.13 \pm 0.06$ by Gibson et al.,[37] but that is still significantly smaller than our $\Gamma_C\Gamma_D = 0.42 \pm 0.14$. Part of this difference may again be traced to a Γ_B less than unity. Martin et al.[16] found evidence in femtosecond studies on both MbO_2 and HbO_2 that there is a transient species formed by photolysis that relaxes at least partly to the ground state of the original bound pair, A, in 1 to 2 ps. This implies that Γ_B is less than unity. They do not attempt to quantify that process; but from their data and our own observations, we doubt that Γ_B could be less than 0.5. Consequently, there must be some additional explanation for the discrepancy. It is possible that the true value of Q lies slightly above the upper limit of the uncertainty range given by Gibson et al.[37] and that the true values of both

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Γ_C and Γ_D are slightly below the uncertainty limits we assigned. On the other hand, it is also possible that there is some small amount of geminate rebinding with a time constant near 2 to 5 ns that has escaped detection by either of our instruments.

Gibson et al.[37] report that for WhMbCO, $Q = 1.00 \pm 0.07$. Earlier work suggested 0.95[18] or 1.0.[40] These results are consistent with the observations of Henry, et al.,[26] who found 4% recombination of geminate pairs of CO to myoglobin with a time constant of 180 ns after photolysis with 10-ns, 532-nm pulses. Our results agree and we conclude that the product of all factors $\Gamma_B\Gamma_C\Gamma_D$ is indeed very close to unity.

In the case of whale myoglobin, approximately 85% of the initially photolyzed NO rebinds on the picosecond time scale. The value of Q cited by Antonini et all.[18] is less than 0.001, while Gibson et al.[37] specify less than 0.01. Either number means that about 15% of ligand rebinding takes place in the nanosecond range. This suggests that the recombination of NO to whale myoglobin from geminate pairs also has a nanosecond as well as a picosecond component. Since Γ_C is measurably smaller in ElMbNO, we would predict that Q for elephant would be even lower than in whale. We know of no measurement. Unfortunately, Q for WhMbNO is already so small as to be difficult to measure precisely; measuring Q for ElMbNO should be even more challenging.

Ours is not the first study to invoke a multistate mechanism to describe the rebinding of ligands to hemoproteins,[7,41] in specific, to sperm whale myoglobin.[6,26,37] Gibson et al.[37] invoke a four-state model, and they attempted an intricate deconvolution procedure to extract fast processes from their data, obtained using 30-ns laser pulses. In the end, however, all their reported rate constants remain slower than 10^8 s^{-1}, except for the unique instance of k_{BA} for WhMbNO (their k_2), for which they infer a value of 1.6×10^{10} s^{-1}, quite close to the slower of the two processes we measure directly in this case. We find processes faster than 10^{10} s^{-1} in all complexes, except WhMbCO.

Although the four-state model is more specific and complex than has been used in the past to treat aqueous solutions of MbL at ambient temperatures, it may still not do full justice to the complexity of protein-ligand interactions. Austin et al.[6] find evidence for multiple intermediates in the dissociation of MbL in glasses at low temperatures. We have some doubt that those intermediates will survive as identifiable, distinguishable species in ambient solution; but the matter is far from settled. It may eventually become possible to correlate features of the picosecond kinetics with aspects of the low-temperature kinetics. In the meantime, our results are accurately accommodated by the four-state model.

Conclusion

We present experimental results which clearly show that the geminate recombination of oxygen and several isocyanides with whale myoglobin is at least a two-intermediate process and can be modeled with a four-state model. The two intermediate states are strikingly different forms of the geminate pair: the contact pair returns with rate constant $k_{BA} > 10^{10}$ s^{-1}, while the separated pair reforms the contact pair with rate constant $k_{CB} \simeq 10^7$-10^8 s^{-1}. Other ligands, specifically CO and NO, do not reveal both processes at current experimental resolution but are easily understood as limiting cases. CO exhibits so little recombination from the geminate pair that we cannot detect it on either time scale; NO exhibits so much fast recombination that there is too little left to detect in the slow process. A variety of ligands, including imidazoles, would be expected to show picosecond recombination reactions with other transition-metal systems. Further experiments are in progress along these lines.

We have also found that steric interactions occur very early in the ligand-binding process. Differentiation between ligands occurs as the ligand enters the protein and not at the iron binding site as has been widely thought. The difference in the overall association rates is determined by ligand size and the rate of ligand motion through the protein into the pocket. After the ligand has diffused to the binding site and orients itself in the necessary configuration with respect to the heme plane, bond formation is very rapid. Except in the case of CO, the rate of bond making is approximately the same for the diatomics as for the bulkier isocyanides. The rate of formation of the separated pair from the contact pair is nearly the same in all cases, again regardless of ligand size. In light of the results concerning the rebinding of isocyanides, it is necessary to further investigate the combination properties of Lewis bases with metal systems.

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Gas-Phase Reactions of $LaFe^+$ with Alkanes

Yongqing Huang and Ben S. Freiser*

Contribution from the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received May 14, 1987*

Abstract: The formation and reactions of $LaFe^+$ with saturated hydrocarbons are reported. Laser-generated La^+ reacts with iron pentacarbonyl to yield predominantly $LaFe(CO)_3^+$, which upon collisional activation yields $LaFe^+$. The reactions of $LaFe^+$ with alkanes are quite different from that of either La^+ or Fe^+. Dehydrogenation is common for the reactions of $LaFe^+$ with alkanes. Dehydrogenation and methane elimination are observed for the reaction of neopentane with $LaFe^+$. Carbon-carbon cleavage and dehydrogenation occur in the reactions of $LaFe^+$ with C_3 and C_4 cyclic alkanes, while dehydrogenation is observed exclusively for cyclopentane, cyclohexane, and methylcyclohexane. The reactivity of $LaFe^+$ is particularly interesting in that several other heterodinuclear ions have been observed to be unreactive with alkanes, although their constituent monoatomic ions are reactive. $D°(La^+-Fe)$ was determined to be 48 ± 5 kcal/mol by photodissociation.

The physical and chemical properties of small metal clusters and their ionic counterparts have been attracting increasing interest over the past decade.[1,2] Both experimental and theoretical research on small metal clusters have been fueled because of their

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